

# Improved Resolution and Very High Sensitivity in MALDI TOF of Matrix Surfaces Made by Fast Evaporation

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Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI TOF) is becoming a standard tool in mass spectrometry in general and protein analysis in particular. Its advantages include high sensitivity, tolerance to buffers, fast data acquisition, and simple and robust instrumentation. Disadvantages have been relatively low resolution and mass accuracy and have mainly been caused by factors related to sample preparation. Here we describe a simple new sample preparation procedure in which matrix and sample handling are completely decoupled. Fast evaporation of a matrix solution based on, e.g., acetone produces a thin layer, presumably consisting of microcrystals. In a second step a drop of analyte solution is deposited on the layer. Some of the features of this new procedure are as follows: (i) there is an improvement in sensitivity; peptides can routinely be analyzed in the attomole range; (ii) salts and impurities are more easily washed off the prepared samples; (iii) sample surfaces become much more homogeneous, allowing very fast data acquisition; (iv) spectra show a more linear mass scale and higher resolution, especially when a reflector is used. In examples, a sensitivity of 5 amol and a resolution of up to 5700 (fwhm) could be achieved for peptides.

Matrix-assisted laser desorption has been introduced as an ionization method for large biomolecules in 1988 by Hillenkamp and Karas.<sup>1</sup> Since then it has become a widespread analytical tool for protein analysis.<sup>2</sup> In their original work Karas and Hillenkamp used nicotinic acid and a frequency-quadrupled Nd:YAG laser. Progress in the past few years has been made with new matrix materials such as cinnamic acid derivative matrices (ferulic acid, sinapic acid,<sup>3</sup> and  $\alpha$ -cyano-4-hydroxycinnamic acid<sup>4</sup>) and 2,5-dihydroxybenzoic acid<sup>5</sup> which could be used with the simpler nitrogen lasers. While other matrices have been discovered for special applications, the above-mentioned four matrices are by far the most commonly used matrices for MALDI MS of proteins and peptides.

In the sample preparation procedure normally used today, a matrix solution is prepared by choosing a solvent in which the matrix material is easily dissolvable such as 30% acetonitrile

in 0.1% aqueous trifluoroacetic acid.<sup>3</sup> The analyte is typically dissolved in aqueous solution. The two solutions are either applied sequentially onto the probe tip of the mass spectrometer or they are mixed in a vial first and the mixture is applied to the tip where it is allowed to dry. In both cases the sample and the matrix must be dissolvable under the same conditions; otherwise, precipitation of one of the components results. As the solvent slowly evaporates, matrix crystals are formed in several places and become easily visible by the naked eye. Experiments by Hillenkamp and co-workers<sup>3</sup> and Beavis and co-workers<sup>6</sup> have shown that the analyte molecules, i.e., the proteins, are distributed throughout the crystals rather than being confined to their surface.

After introduction of the sample into the mass spectrometer, it is often necessary to find "good spots" on the probe tip, at least in samples with a low abundance of analyte or with high levels of contaminants. In systems equipped with microscopic in situ observation it can be seen that the good spots coincide with particular crystals which apparently have incorporated an optimal number of analyte molecules.

There are obvious disadvantages of this "conventional" preparation method in addition to the need of having matrix and analyte dissolved in the same medium. The resulting surfaces can be quite inhomogeneous leading to signal variations over the surface of the target. Because of the inhomogeneous crystallization process there is little correlation of the signal intensity with the amount of sample analyzed. Furthermore, the different amount of matrix present at various spots will lead to the ejection of changing amounts of neutral matrix material from which the analyte ions are extracted. Thus initial conditions of analyte ions are not well defined which can lead to decreased resolution.

Several strategies have already been tried to alleviate some of the above problems and to make the sample preparation method more reproducible. Weinberger et al. have tried drying the mixture of sample and matrix in vacuum to achieve smaller and more homogeneous crystals.<sup>7</sup> This method has apparently lead to improved results in some cases, but it was not generally useful in our hands and has not become widespread. The same investigators have also experimented with a two-step crystallization procedure in which they first produce a layer of analyte and matrix cocrystallizes on the target, wipe it off, and subsequently grow a second layer of analyte matrix

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(1) Karas, M.; Hillenkamp, F. *Anal. Chem.* 1988, 60, 2299.

(2) Hillenkamp, F.; Karas, M.; Beavis, R. C.; Chait, B. T. *Anal. Chem.* 1991, 63, 1193 A.

(3) Beavis, R. C.; Chait, B. T. *Rapid Commun. Mass Spectrom.* 1989, 3, 432-435.

(4) Beavis, R. C.; Chaudhary, T.; Chait, B. T. *Org. Mass Spectrom.* 1992, 27, 156-158.

(5) Strupat, K.; Karas, M.; Hillenkamp, F. *Int. J. Mass Spectrom. Ion Processes* 1991, 111, 89.

(6) Beavis, R. C.; Bridson, J. N. *J. Phys. D* 1993, 26, 442.

(7) Weinberg, S. R.; Boersen, K. O.; Finch, J. W.; Robertson, V.; Musselman, B. D. Proceedings of the 41st ASMS Conference on Mass Spectrometry and Allied Topics; San Francisco, CA, 1993; pp 775a-775b.

material on top of it,<sup>8</sup> thus using the remains of the first layer as a seed for the second layer. An improvement in resolution and quantitation was noted for this procedure.

Xiang and Beavis recently published a similar sample preparation method and noted that it allows the use of much more contaminated samples than was possible previously.<sup>9</sup> The authors prepare a seed layer by drying matrix solution and crushing it mechanically on the probe tip. Subsequently, they use the conventional method described above to apply the sample.

In all these methods analyte and matrix cocrystallize and analyte molecules are embedded in the bulk of the crystal material.

Hutchens and Yip have tried to covalently bind a monolayer of matrix material on the surface of the probe tip and to dry the sample on top of this surface.<sup>10</sup> While this method would seem to be ideal for surface homogeneity and efficient use of the sample, it is difficult to see how the required embedding of the analyte molecules into the matrix molecules could be achieved. There has, indeed, been no confirmation of the results of that method from other laboratories.

We here describe a newly developed sample preparation procedure, which completely decouples the matrix handling and surface preparation from sample handling and which has numerous advantages over the conventional procedure. In the new procedure, matrix solution is applied to the probe tip of the mass spectrometer in a highly volatile solvent, e.g., acetone, to obtain very fast evaporation of the solvent. This leads to the formation of a dense, flat, and thin film presumably consisting of very small crystals of matrix. A small volume of analyte solution is then placed on top of the matrix surface, and the liquid is allowed to evaporate slowly. The only constraint on the analyte solution is that it must not completely redissolve the matrix surface but only the outermost layer. We speculate that this layer is then doped with analyte molecules.

Some of the most important advantages that these surfaces have over the conventional preparations are better sensitivity, washability, surface homogeneity, and energy distribution of desorbed ions. Surface preparation is simple, involving only a single pipetting step.

In this paper we describe the preparation of homogeneous matrix surfaces and show examples that illustrate how analyses are improved as a result of the new sample preparation procedure.

## EXPERIMENTAL SECTION

**Materials.** Substance P, RNase A, and chicken lysozyme were purchased from Sigma Chemicals Co., St. Louis, MO. Human insulin was donated by Novo-Nordisk, Gentofte, Denmark. Bovine pancreatic trypsin was from Boehringer, Mannheim, Germany. Synthetic peptides were made by M. LeBreton and R. Jakob in our group at the European Molecular Biology Laboratory.

Ferulic acid, sinapic acid,  $\alpha$ -cyano-3-hydroxy-*trans*-cinnamic acid, and  $\alpha$ -cyano-4-hydroxy-*trans*-cinnamic acid were

from Sigma Chemicals Co., St. Louis, MO. Pure water was made with an ElgaStat from Elga, High Wycombe, UK. Acetone, acetonitrile (MeCN), methanol (MeOH), acetic acid (Hac), and trifluoroacetic acid (TFA) were obtained from Labscan Limited, Dublin, Ireland. Formic acid was from Sigma Chemicals Co., St. Louis, MO. All chemicals were of analytical grade.

**Mass Spectrometry.** All mass spectra were obtained on a Bruker reflex TOF mass spectrometer (Bruker-Franzen Analytik, Bremen, Germany). Pressures in the source region and the reflector region were below  $10^{-6}$  and  $10^{-7}$  mbar, respectively. The data acquisition system that normally comes with the instrument was replaced by a LeCroy 9450A 400 megasamples/s digital storage oscilloscope (LeCroy Corp., Chestnut Ridge, NY) from where single-shot spectra were transferred to a Macintosh Quadra 950 computer (Apple Computer Inc., Cupertino, CA) via a National Instruments NI DAQ GPIB controller board (National Instruments, Austin, TX).

Control of data-acquisition parameters, the transfer and subsequent averaging of spectra as well as all further data processing was carried out using the computer program LaserOne developed in-house.<sup>11</sup>

To enable precise adjustment of acceleration and reflector potentials, a Probus III 16-bit digital-to-analog-converter (FUG Elektronik, Rosenheim, Germany) was used to control the outputs of two 35 kV high-voltage power supplies (FUG Model HCN 140M-35000) from the Macintosh computer.

Low-mass ions could be deflected by a pulsed electrical field orthogonal to the flight path. The pulsed time window was chosen in LaserOne and then controlled by a Stanford Research Systems Model DG535 digital delay pulse generator (Stanford Research Systems Inc., Sunnyvale, CA), which was triggered by the acquisition start trigger of the mass spectrometer.

**Preparation of Matrix Surfaces.** Matrix materials are dissolved in acetone containing 1–2% pure water or 0.1% aqueous TFA. The concentration of matrix ranges between the point of saturation and one third of that concentration. Approximately 0.5  $\mu$ L of matrix solution is deposited at the center of a stainless steel probe tip. Because acetone evaporates rapidly from the pipet tip, the transfer must be rapid, i.e., less than a few seconds. On deposition the liquid spreads quickly by itself, and the solvent evaporates almost instantaneously. At room temperature and when using acetone as the solvent, spreading and evaporation should not take longer than 1–2 s. The resulting matrix surface can be checked for homogeneity; apart from a thickening at the rim, no inhomogeneity should be visible by eye or light microscopy.

The preparation procedure just outlined need not be followed very carefully to prepare microscopically sized matrix crystals. But a pivotal aspect seems to be the fast spreading and evaporation of the solvent from the matrix solution. Methanol could be used instead of acetone, and also the few percent water or aqueous acid could be omitted, although these changes made the surface preparation slightly more difficult.

(8) Weinberg, S. R.; Boernsen, K. O. Kyoto '92 International Conference on Biological Mass Spectrometry, Sept 20–24, 1992.

(9) Xiang, F.; Beavis, R. C. *Rapid Commun. Mass Spectrom.* 1994, 8, 199–204.

(10) Hutchens, T. W.; Yip, T. T. *Rapid Commun. Mass Spectrom.* 1993, 7, 576.

(11) LaserOne was written using object oriented programming in Think C (Symantec Corp., Cupertino, CA) by M. Mann and P. Mortensen, EMBL, Heidelberg, Germany.

The above procedure applies to large and flat probe tips. In systems which use small or curved probe tips slight alterations may be necessary to achieve homogeneous matrix surfaces. The amount of matrix solution and its concentration can be used to adapt the procedure to the system used. On stainless steel 0.2  $\mu\text{L}$  results in a small spot of a few millimeters in diameter, and 1  $\mu\text{L}$  results in a larger spot of ca. 10 mm diameter. In some experiments with probe tips having a 1.3 mm diameter and thus a  $\sim 10$  times smaller area than the probe tips of the Bruker instrument the best matrix surfaces were made when using 10 times lower concentration of matrix in acetone and applying the same volume of solution to the tips. The optimal choice of concentration may also depend on the material of the probe tip surface.

**Loading and Washing of Samples.** Analyte molecules can be dissolved in any solution provided that the solution does not completely redissolve the crystals. The sample is simply placed on top of the matrix surface and allowed to dry either by itself or in a stream of nitrogen. Best results are obtained when the analyte droplet is placed on the homogeneous part of the matrix surface and not on the rim.

After the droplet has dried a first mass spectrometric analysis can be performed on the sample. Most often, however, it is advantageous to "wash" the sample by placing a large droplet of 5–10  $\mu\text{L}$  of water or dilute organic acid on top of the sample spot. The liquid is left on the sample for 2–10 s and is then shaken off or blown off by pressurized air. The procedure can be repeated once or twice. The washing liquid should be as free as possible of alkali-metal salts and should be neutral or acidic (i.e., 0.1% TFA).

Analyte solutions which are basic or contain a high percentage of organic solvents can completely redissolve the matrix surface. Such damage can easily be seen either by eye or by light microscopy as areas on the target where the metal is visible. This problem can be remedied by diluting the sample with aqueous acid. Alternatively, a small droplet of, e.g., 1% aqueous TFA can be placed on the surface. The analyte solution is then added to this droplet and the mixture which is both acidic and sufficiently aqueous is allowed to dry in the normal fashion.

Successful analyses have been performed with analytes dissolved either in pure water, 0.1–2% aqueous TFA, 1–10% aqueous acetic acid, 1–70% aqueous formic acid, water/acetonitrile, or aqueous acid/acetonitrile. Analyte concentrations have ranged between 10 amol/ $\mu\text{L}$  and 200 pmol/ $\mu\text{L}$ .

All probe tips were cleaned extensively immediately prior to analyses undertaken to test the sensitivity of the two sample-preparation procedures. After several spot tests proved it effective, the preferred cleaning procedure became sonication of the tips for approximately 15 min in a mixture of equal volumes of formic acid, ethanol, and water.

## RESULTS AND DISCUSSION

**Characteristics of Matrix Surfaces.** Figure 1 shows a photograph of a surface formed of  $\alpha$ -cyano-4-hydroxycinnamic acid by fast evaporation from acetone solution (Figure 1a). This matrix results in a featureless flat surface with a color which tends to be yellowish. Preparations with ferulic and sinapic acid look similar but have slightly different colors; ferulic acid becomes slightly pink, and sinapic acid is almost

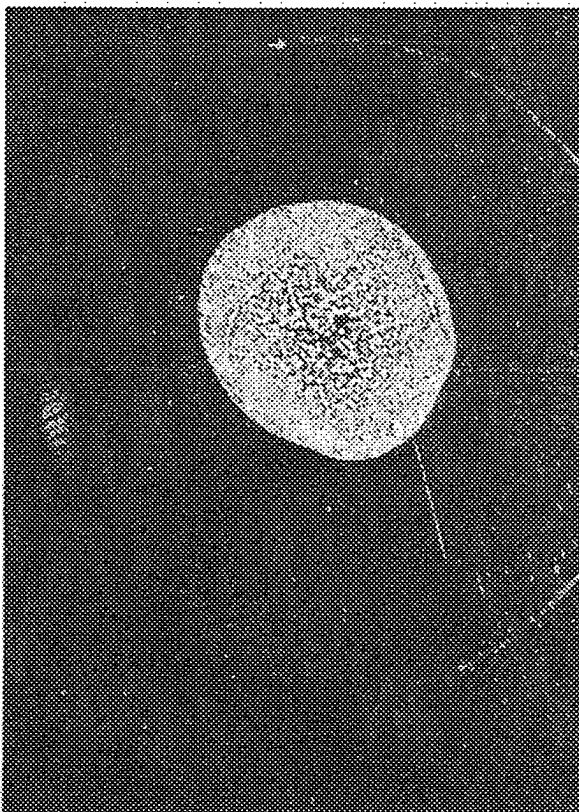
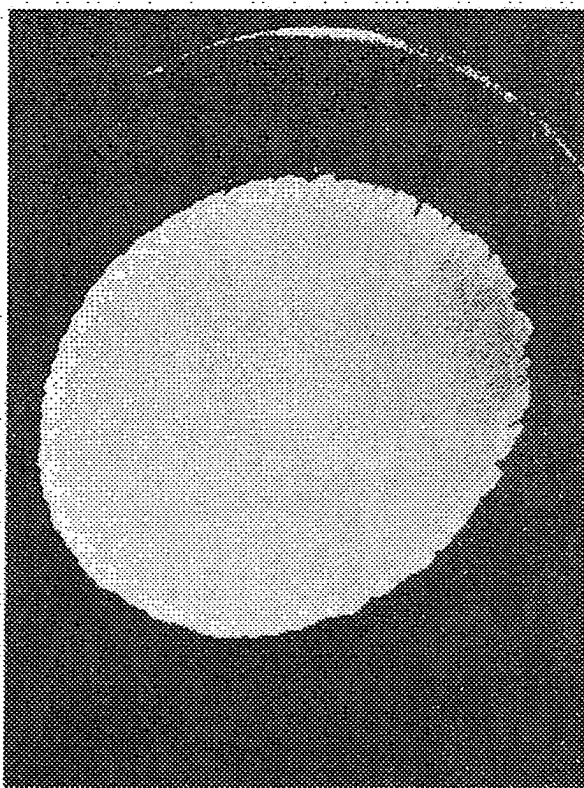


Figure 1. Photographs of 0.5  $\mu\text{L}$  of  $\alpha$ -cyano-4-hydroxycinnamic acid solution evaporated on a stainless steel probe. The probe tip is polished and has a diameter of 8 mm: (a, top) matrix surface prepared as described in experimental; (b, bottom) crystals formed from a saturated solution of matrix in 33% aqueous acetonitrile.

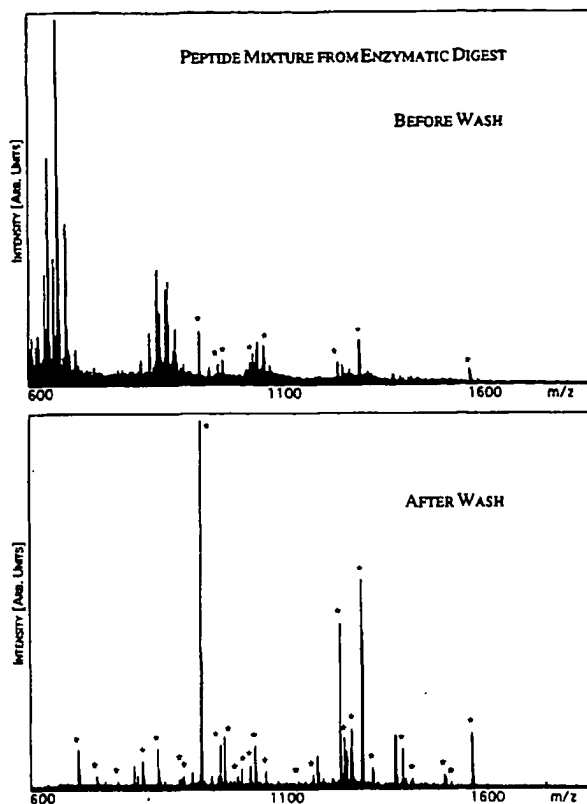
white. This was not the case for  $\alpha$ -cyano-3-hydroxycinnamic acid, which creates a very thin transparent film that appears green due to light diffraction. Also shown is a preparation of the same volume of matrix applied in the conventional way, i.e., in partly aqueous solvent (Figure 1b). In that case the matrix material is concentrated in few large crystals which are far apart.

The film thickness has not been measured directly for any of the matrix surfaces. However, light microscopy at 120 times magnification did not show any discernible features in the surface, in marked contrast to the conventional preparation method. Preliminary results with atomic force microscopy (data not shown) indicated that the surface consists of closely spaced flat topped particles with sizes in the 1  $\mu\text{m}$  range. From the data it could not be decided if the particles were of amorphous or crystalline structure. At this point we assume that the particles are microcrystals. A surface prepared in the conventional way showed a roughness at least 10–100 times larger.

**Surface Homogeneity Assessed by Mass Spectrometry.** A major advantage of the matrix surface preparation was the homogeneity of the sample surface. Data acquisition was made faster and easier by the fact that all spots on the surface resulted in similar spectra at the same laser irradiance. No or little search for useful crystals was necessary, and the outcome of the first few single-shot spectra of a sample usually revealed whether a MALDI MS analysis would be successful.

Matrix surfaces prepared by fast evaporation exhibit irradiance threshold characteristics resembling those of ordinary sample preparations. However, when using small amounts of sample and an irradiance just above threshold, we found that a given spot typically would yield only around 20 good mass spectra. Then one either had to move to a new spot next to the used one or remain at the same spot and increase the irradiance. With the laser beam focused to an area of approximately 100  $\mu\text{m} \times 200 \mu\text{m}$ , we found it easiest to keep moving to new spots, a strategy which also was necessary for analyses near the detection limit. In a few test cases with abundant analyte, i.e., 1 pmol or more, the irradiance could gradually be increased to obtain up to 2000 good single-shot spectra from the same spot before the matrix was used up. One possible explanation of this finding is that due to inhomogeneity of the laser beam the laser irradiance exceeds the threshold for ion production in only a small part of the total irradiated area. As the laser irradiance is increased, new areas of the surface exceed the threshold and can thus emit ions.

From use of the technique in everyday analyses (as estimated from Edman degradation results) it is our impression that the amount of sample and the signal from the LDMS apparatus are correlated more closely than they are in the conventional preparation technique. Such a behavior would be expected from the decreased shot-to-shot variability and the decreased variability across the surface. Furthermore, many crystals are illuminated in every laser shot. Therefore, even if individual crystals were quite different (as is the case for the conventional preparation method), the statistical average obtained for any laser position is well defined and



**Figure 2.** Reflector mass spectra of a peptide mixture before (upper panel) and after washing (lower panel). The matrix surface was made of  $\alpha$ -cyano-4-hydroxycinnamic acid (0.6  $\mu\text{L}$  20  $\mu\text{g}/\mu\text{L}$  in acetone with 1% water). Subsequently, 0.3  $\mu\text{L}$  of peptide solution<sup>13</sup> in 20% acetonitrile was applied. The wash was carried out by depositing 10  $\mu\text{L}$  of 0.1% TFA on the sample spot and the liquid was shaken off after  $\sim 5$  s. The washing procedure was repeated once. Peaks from tryptic peptides are marked by asterisks. All peptide peaks are isotopically resolved.

roughly constant over the surface. While MALDI is still not a quantitative method, some progress has thus been made with the new surface preparation technique.

**Washability.** The removal of impurities—especially alkali-metal ions—is known to be important in any kind of mass spectrometry of proteins and peptides. For example, extensive washing of prepared samples immediately prior to analysis has been extremely useful in  $^{252}\text{Cf}$  plasma desorption mass spectrometry where nitrocellulose is used as backing material.<sup>12</sup> But such washing is not normally possible for samples in MALDI made by the conventional sample preparation procedure. Matrix crystals are large and far apart, and they have little interaction with one another and little adhesion to the probe tip surface. Thus entire crystals wash away easily. In matrix surfaces prepared by fast evaporation, on the other hand, the microcrystals somehow stick to each other and the matrix as a unit has strong adhesion to the probe tip surface.

We have found that the matrix surfaces can be washed extensively without damage. One efficient washing procedure was to place a large droplet of dilute organic acid on the surface after the analyte solution had dried completely and then to blow off the droplet by pressurized air after a few seconds (see description in the Experimental Section). Even on probe tips with a very smooth surface, e.g., polished stainless

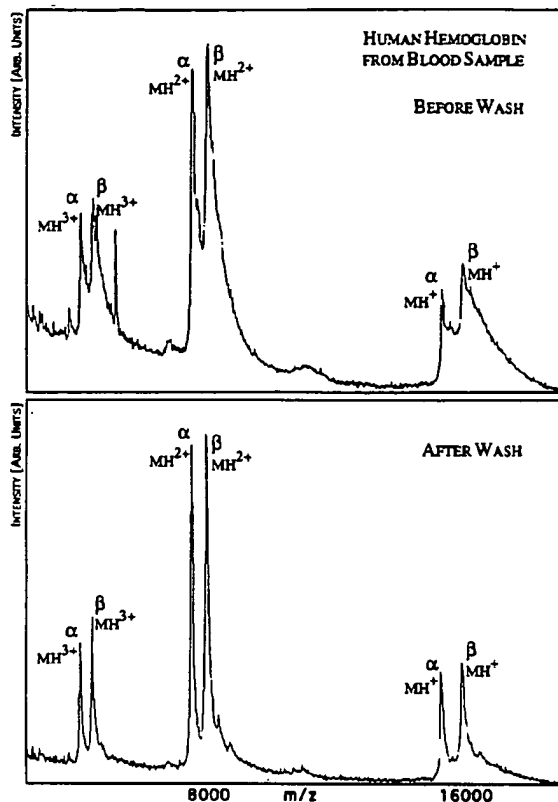
(12) Nielsen, P. F.; Klarskov, K.; Højrup, P.; Roepstorff, P. *Biomol. Environ. Mass Spectrom.* 1988, 17, 355–362.

steel, the matrix surface would in most cases remain intact after repeated washing steps. Apparently, the only amount of matrix that disappeared was that dissolved in the volume used for washing. As the cinnamic acid derivative matrices used in this study are poorly soluble in pure water as well as in the dilute organic acids used in this study, this amount is assumed to be very small. The matrix compounds are much more soluble in basic solutions. Therefore it is advisable to use acidified water (e.g., 0.1% aqueous TFA, 10% aqueous acetic acid or formic acid) for the washing procedure in order to maintain a low pH in cases where the analyte solution originally contained alkaline impurities.

The importance and efficiency of washing samples is illustrated in Figure 2 which shows the MALDI spectra of a relatively dirty peptide mixture obtained by tryptic digestion of a human protein isolated by 2D-gel electrophoresis.<sup>13,14</sup> The two spectra were obtained from the same sample, where the upper panel shows the results before and the lower panel the results after washing the sample as described above. The signal-to-noise ratio of all identified tryptic peptide peaks (marked by asterisks) was much improved by the washing step. In addition, all background and impurity peaks were removed or greatly reduced in intensity. As a consequence, the data interpretation was more reliable and much simpler, which is very important in peptide mapping. Absolute intensities of the measured peptides typically increased rather than decreased after one washing step. However, we did find washing of targets a disadvantage in analyses of very small amounts of analyte. In such cases as in all critical applications the recommended procedure is to take spectra before and after each washing step.

In MALDI spectra of proteins with a molecular weight between 5 and 20 kDa we generally observed that washing the prepared targets led to notable reduction in peak tailing in addition to the expected improvements in S/N ratio. In Figure 3 spectra have been obtained from the same sample before (upper panel) and after the washing step (lower panel). The spectra are of human hemoglobin  $\alpha$ - and  $\beta$ -chain that was obtained directly from a highly diluted blood sample where the erythrocytes were lysed by sonication (see figure legend). A considerable improvement in S/N ratio is evident; but more importantly, the prominent high-mass tailing disappeared and the mass accuracy could be improved.

**Sensitivity.** A primary difference between the conventional preparation and the new preparation technique is the size of the crystals produced. Because the crystals are not substantially redissolved and no additional matrix material is employed, proteins can only be located in the outer crystal layers. The surface area of crystals should be very large in the current method. Since desorption must occur from the outer layers of the crystals and since this is where the analyte molecules are embedded, we expect an even higher sensitivity for the surface technique than for MALDI using the conventional preparation technique. Normally the amount



**Figure 3.** Linear TOF mass spectra of human hemoglobin  $\alpha$ - and  $\beta$ -chains obtained before (upper panel) and after washing (lower panel). The hemoglobin was obtained from a fresh blood sample of 0.3  $\mu$ L that was diluted to 200  $\mu$ L in water and sonicated for  $\sim$ 7 min. This solution was then further diluted by a factor of 15 in 10% formic acid, and 0.5  $\mu$ L of that solution was added to a matrix surface of  $\alpha$ -cyano-4-hydroxycinnamic acid. The wash was undertaken as described in Figure 2.

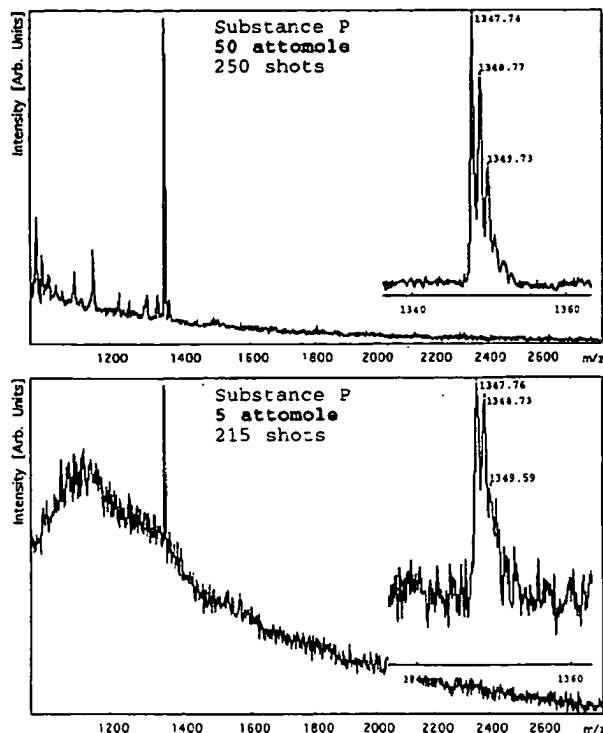
of sample used in MALDI MS analyses is in the low-picomole to high-femtomole range, and in a few reports the sensitivity of MALDI has been demonstrated to be in the low-femtomole range.

With the new sample preparation method described here, we found that sensitivity has increased by about 2 orders of magnitude in comparison to the conventional preparation method. Absolute detection limits still depend highly on sample and instrument specific features, however. Analyses with excellent mass spectrometric results were made on ca. 100 amol of standard proteins up to mass 14 kDa (e.g., chicken lysozyme). The only compound for which we tried to determine the limit of detection was substance P (monoisotopic,  $M_r$  1347.736), chosen because it is a much used standard peptide. Figure 4 shows the reflector TOF mass spectra of 50 and 5 amol substance P with inserts of the molecular ion regions. Data acquisition in the case of 50 amol substance P was straightforward, and all single-shot spectra were averaged without selection. In the case of 5 amol of substance P, on the other hand, unselective averaging was possible only in roughly three-quarters of all analyses. All attempts to detect 0.5 amol failed.

It is not straightforward to use these extremes of sensitivity in routine analysis. Although probe tips could be cleaned satisfactorily and there was thus no "carry-over" problem, it

(13) The electrophoresis was carried out by Hanne Holm Rasmussen, Department of Medical Biochemistry, Aarhus University, Denmark. After electrophoresis the protein was electroblotted onto a poly(vinylidene fluoride) (PVDF) membrane where it was digested with trypsin (by Ejvind Mørtz, Department of Molecular Biology, Odense University, Denmark). Similar work is described in detail in the next reference.

(14) Rasmussen, H. H.; Mørtz, E.; Mann, M.; Roepstorff, P.; Celis, J. E. *Electrophoresis* 1994, 15, 406-416.

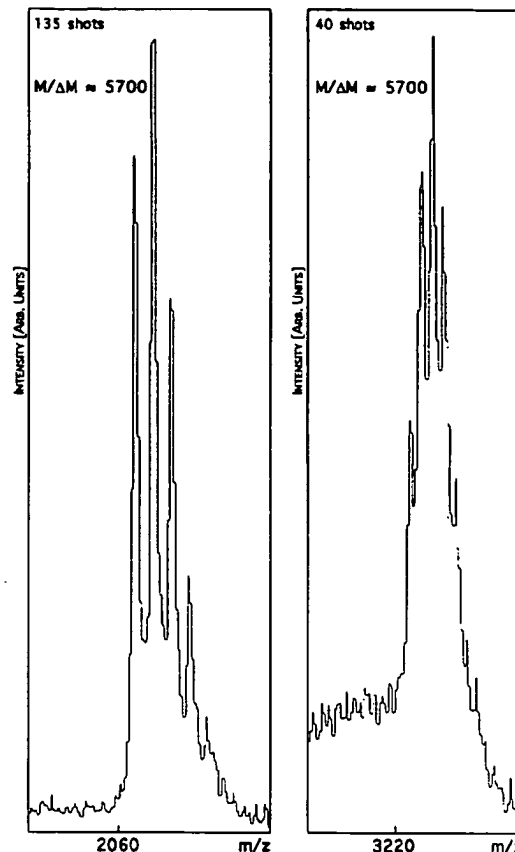


**Figure 4.** Reflector mass spectra of substance P (seq.: RPKPQQFF-GLM-NH<sub>2</sub>, monoisotopic,  $M_r$  1347.736). Matrix surfaces of  $\alpha$ -cyano-4-hydroxycinnamic acid were used for both spectra. Volumes of 0.5  $\mu$ L of substance P solution (10% acetonitrile in 0.1% aqueous TFA) were added. For the upper spectrum the peptide concentration was 100 amol/ $\mu$ L and for the lower it was 10 amol/ $\mu$ L. Both concentrations were obtained from a serial dilution.

was very difficult to obtain spectra of compounds in the low-attomole range without dominance of peaks from impurities. Despite great precaution, solutions tended to become contaminated either with various peptides or with polymer detergents such as poly(ethylene glycol). Thus we found the lower limit of the routine working range for matrix surfaces prepared by fast evaporation to be in the high-attomole range. Our general approach to analyses of most biological samples became to dilute them with high-purity solvents to an estimated concentration of about 25 fmol/ $\mu$ L and apply 0.3–1  $\mu$ L onto the matrix. This procedure has the added advantage that contaminants are diluted to usually harmless levels.

At the other extreme of sample amount we found in analyses of various synthetic peptides that several picomole of peptide could be loaded onto the surfaces with no degradation in performance. Thus the dynamic range of the technique is at least  $10^5$  and no special precaution has to be taken to find the correct sample amount to analyze. Applying more than 100 pmol of analyte could lead to reduced mass resolution and larger shot-to-shot spectrum variations.

**Resolution.** One very important practical consequence was that matrix surfaces allowed much improved mass resolution to be obtained effortlessly. This was especially striking in reflector mass spectra where under similar conditions resolution increased at least by a factor of 2. Thus, we obtained mass resolutions up to 5700 (fwhm) for medium-sized peptides. The molecular ion regions from two synthetic peptides measured with such resolution are shown in Figure 5. There



**Figure 5.** Molecular ion region of the reflector TOF mass spectra of two synthetic peptides. The left panel is from the peptide KEKITQY-IYHVLNGEIL, monoisotopic,  $M_r$  2061.139, and the right panel is from the peptide HLENMGSHDIVDGNHRLTGLIWTIILR, monoisotopic,  $M_r$  3222.732. Matrix surfaces of  $\alpha$ -cyano-4-hydroxycinnamic acid were used for both samples and they were both washed three times with 8  $\mu$ L volumes of water that were shaken off shortly after their addition.

have been reports of similarly high resolution by other groups<sup>15</sup> and even higher ones with special instrumentation (e.g., two reflectors<sup>16</sup>). We have also observed high resolution from clean peptide samples using the conventional preparation technique. However, these results tended to be isolated instances and have been obtained for standard peptides and seldomly for mixtures. In contrast, with the present method a mass resolution of 2500 is standard even for difficult samples and very complex mixtures. Thus, in the important mass range of tryptic peptides, i.e., up to a mass of about 2300 Da, isotope resolution of all components can be achieved routinely.

**Mechanistic Aspects.** Neither the internal energy nor the initial kinetic energy of ion species can be measured directly with the Bruker reflex mass spectrometer. However, several findings indicate that the ions desorbed from surfaces prepared by fast evaporation are cooler and have more uniform kinetic energies than those produced in the desorption/ionization from ordinary sample preparations.

It is common, that the peak of an ion species shifts toward longer time-of-flight and becomes broader when the irradiance is increased from being just above to being well above threshold

- (15) Ingendoh, A.; Karas, M.; Hillenkamp, F.; Giessmann, U. *Int. J. Mass Spectrom. Ion Processes* 1994, 131, 345–354.
- (16) Cornish, T. J.; Cotter, R. J. *Rapid Commun. Mass Spectrom.* 1992, 6, 242.

or when the laser beam is scanned across a target and strikes better absorbing matrix crystals. These effects were still observed with the matrix surfaces, but they were much less pronounced than usual.

Generally, ions showed less metastable fragmentation in reflector measurements when using the new matrix preparations. For example, in the spectrum of Figure 2 there were no metastable peaks (identified by a factor  $\sim 3$  lower resolution than stable peptides).

Research by several groups has pointed to the pivotal role of the matrix plume in the desorption event.<sup>17-20</sup> Briefly, it is thought that ions have to be pulled out of the plume or cloud generated by a laser desorbed volume of matrix material. The collisions that the ions experience in this process can lead to an energy deficit and impart internal energy which may lead to metastable decay. Furthermore, differences in flight times could be introduced between different species, depending on where they are released from the plume.

The absence of such observations with the current technique, i.e., very little metastable decay, little dependence of flight times on irradiance, little dependence of resolution on irradiance, is consistent with the assumption of little plume generation. Less plume generation would be expected since peptides merely have to be desorbed from the outer layers of the crystals.

Preliminary experiments indicated that the mass scale followed the square root law much more accurately than samples prepared in the conventional way. This feature promises much higher mass accuracy than was previously possible. As an example, the mass of substance P in Figure

4 was measured to much better than 0.1 Da without the use of an added standard. Improvements in mass accuracy will be investigated separately.<sup>21</sup>

## CONCLUSION

The new matrix surface sample preparation method has numerous important advantages over the conventional dried-droplet method. The procedure is simpler, allows matrix surfaces to be prepared in advance, and, most importantly, results in homogeneous distribution of matrix and analytes. All these factors enable easier and faster data acquisition and thus lead to considerable time savings. The matrix surfaces are easily washed to remove buffers, salts, and other impurities that would normally hinder useful data to be obtained from conventional samples. In addition, significantly improved sensitivity and resolution are obtained effortlessly as direct consequence of using matrix surface preparations instead of the conventional preparations.

When compared to the dried-droplet samples, these relative improvements have been observed on more than 10 different MALDI time-of-flight mass spectrometers in eight different laboratories. Nevertheless, the absolute limits of performance, especially in terms of sensitivity and resolution, depend very much on the individual instruments.

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